THE USE OF ADJUVANTS IN STUDIES ON INFLUENZA IMMUNIZATION

I. Measurements in Monkeys of the Dimensions of Antigenicity of Virus–Mineral Oil Emulsions*

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Rather early in studies on influenza immunization in animals as well as in man, it was realized that immunizing effect, as reflected in antibody response induced by the vaccines employed, was limited and of relatively short duration. For these reasons (1) a variety of methods were explored for enhancing and prolonging the protective effect induced by vaccination (2–7). None, however, provided such striking results as those obtained in studies in animals reported by Friedewald (6), and in studies in man reported by Henle and Henle (7). These investigators used vaccines consisting of virus in water-in-oil emulsions in the manner developed in recent years by Freund (8).

Although the immunologic data derived by Friedewald and the Henles were outstanding, in comparison to the effects induced by vaccines consisting of virus in an aqueous medium alone, the immunologic success was blighted by the occurrence of undesirable reactions at the site of inoculation. The problem remaining after these experiences seemed merely to be one of reducing or eliminating the untoward local reaction while retaining the immunologic advantage. That this could be accomplished was evident from observations made, in the course of certain other studies (9), when it was found that rather large doses of water-in-oil emulsions without acid-fast bacilli could be inoculated into monkeys without untoward local reactions. In the studies referred to reagents were used which were different from those employed by Friedewald and the Henles and the inoculum was introduced *intramuscularly rather than subcutaneously*. When influenza virus vaccine was prepared in this particular water-in-oil emulsion and administered to monkeys intramuscularly, it too could be given without producing undesirable effects at the inoculation site.

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The absence of reactions and the high levels of antibody induced in animals inoculated with virus emulsified with the agents to be described prompted a series of experiments to determine the degree and duration of the immunologic effect; for theoretical as well as practical considerations it was of interest not only to determine the height and persistence of the increased antibody titer, but to know as well how small a quantity of antigen when incorporated with the adjuvant would still be effective in inducing antibody formation. It seemed important, too, to study the course of development of antibody activity as measured by different methods, *i.e.*, hemagglutination inhibition, virus neutralization, and complement fixation, to gain further information regarding the relationship among these three activities of the serum of vaccinated animals.

The results to be presented will indicate that the vaccine employed could be diluted approximately 100,000-fold and, when incorporated with mineral oil, was still effective in inducing antibody formation; vaccine without mineral oil was ineffective in this respect when diluted 100-fold; and, it was found that the antibody activities measured by the three different methods develop and decline at different rates.

Materials and Methods

Vaccine.—The vaccine used in these studies contained the PR8 strain of influenza virus type A; it had been prepared by a manufacturer of biologicals especially for use in field studies by the Commission on Influenza (10). The method of preparation involved sedimentation by Sharples centrifugation of virus from allantoic fluid of infected chick embryos; the virus was then resuspended to a concentration of 400 CCA[1] units per ml. (11). Infectivity was destroyed by treatment with 1:2000 dilution of formalin. When used in the experiments to be described, the vaccine had been stored at 1–4°C. for approximately 2 years.

Emulsification with Mineral Oil.—The mineral oil used in these experiments is known as bayol F and was obtained from Esso Standard Oil Company, Philadelphia. The emulsifying agent is referred to as arlacel A; the latter is the trade name for mannide monooleate and was supplied by The Atlas Powder Company, Wilmington, Delaware. Both reagents have been used by Freund and his associates (12) and in the studies referred to above (9). In the initial experiments, the oil and the emulsifying agent were sterilized separately in the autoclave, for 15 minutes, at a pressure of 15 pounds and at 120°C. Subsequently these reagents were mixed prior to sterilization in a ratio of 9 parts of bayol F to 1 part of arlacel A. Undiluted vaccine or the desired dilution made in buffered physiologic salt solution containing 0.05 M phosphate buffer, pH 7.3, was mixed with the oil and emulsifying agent in the ratio of 10:9:1. The aqueous phase was added dropwise to the other two reagents, contained in a mortar, and blended with the pestle. Homogenization was accomplished by repeated filling and forcible ejection from a 10 cc. Luer lock syringe fitted with an 18 gauge needle, 1/4 inch in length. It usually required 10 to 15 minutes of mixing to effect complete emulsification; this was indicated when a drop of the viscous mixture placed on the surface of water in a beaker or paper cup remained discrete. The mixture was inoculated usually within an hour after preparation although this is not necessary since the emulsion and the antigen contained therein are quite stabile. Emulsification for the preparation of material tested in humans, to be reported separately, was effected in a closed system by means of a syringe and 17 to 18 gauge needle inserted into the diaphragm of a vaccine vial.

[1]Chicken cell (red) agglutination.

Experimental Animals, Methods of Inoculation, and Bleeding.—Rhesus monkeys, weighing from 5 to 8 pounds, used in these studies were healthy, non-paralyzed survivors of studies on poliomyelitis. In preparation for injection, the skin over the calf of the left leg was shaved and cleansed with tincture of iodine. A 22 gauge needle, 1½ inches in length, was fitted to a 2 ml. Luer lock syringe for injection of the 1 ml. dose and to a 0.5 ml. tuberculin syringe which was used for injection of the 0.1 ml. dose. In each instance, approximately 0.5 ml. of air was drawn into the syringe so as to force the inoculum through and avoid loss of material that would otherwise remain in the needle. Groups of three or four monkeys were inoculated with each mixture of emulsified vaccine or vaccine in saline; observations were made for evidences of local reaction and blood samples were obtained at intervals indicated in the text. Bleedings of 5 to 10 ml. were made from the femoral vessels; animals were anesthetized lightly with ether or by intraperitoneal injection of 5 per cent sodium pentobarbital, employing a dose of 0.2 ml. per pound. All serum samples were handled aseptically and stored at 1–4°C.

Methods Employed for the Serologic Tests.—In the investigations here reported, immunologic methods embodying three different principles were used: (*a*) hemagglutination inhibition, (*b*) virus neutralization *in ovo*, and (*c*) complement fixation.

Hemagglutination Inhibition.—The method for determining the quantity of hemagglutination-inhibiting substance has been described (13). The virus used as antigen was prepared from allantoic fluid of embryonated eggs, infected with the same seed virus used in the preparation of the vaccine. Eggs were inoculated on the 10th or 11th day of incubation and the antigen derived after 48 hours of further incubation at 35°C. Storage was at 1–4°C. and the titer of hemagglutinating activity determined immediately prior to and at the time of its use in the serologic test. Sera tested by this method were heated at 56°C. in a water bath for 30 minutes; serial twofold dilutions in physiologic solution of sodium chloride were made using a separate pipette for each dilution. Dilutions were prepared in test tubes and 0.5 ml. of each transferred to a plastic plate (14). To each dilution was then added 0.25 ml. of the antigen containing a sufficient amount of material so that 4 hemagglutinating units were present in the final mixture and to the serum-virus mixture was then added 0.25 ml. of 1 per cent chicken red blood cell suspension. The end-point was considered to be the highest dilution of serum that completely inhibited the hemagglutinating effect of the virus. The results are expressed here as the final dilution of serum in the mixture. The sera from monkeys in any one vaccinated group were tested simultaneously and when it was necessary to relate the results of tests on different days, a pool of human immune serum was included as a standard for comparison.

Virus Neutralization Test in Ovo.—For this purpose, a pool of virus contained in allantoic fluid of infected chick embryos was distributed in glass ampules, sealed, and stored at −70°C. Prior to use in the serologic test the 50 per cent embryo infectious dose (EID_{50}) was determined; infection in the embryo was indicated by the presence of hemagglutinin in allantoic fluid of embryos inoculated by this route. Serum dilutions were made in beef heart infusion broth containing 100 units of penicillin and 1000 μg. of streptomycin per ml. to reduce the likelihood of bacterial contamination; sera with low levels of antibody, as determined by preliminary test, were diluted in fourfold steps; those with high levels of antibody were diluted in tenfold steps. Serum-virus mixtures consisting of equal volumes (0.5 ml.) of the respective dilutions of serum and of diluted virus containing approximately 2000 EID_{50} were incubated in a water bath at 37°C. for 30 minutes prior to inoculation of 0.1 ml. volumes into the allantoic sac of each of five 10 day old embryos. Incubation was continued at 35°C. for 2 more days and the eggs candled to recognize embryos that had died following inoculation; after refrigeration at 4°C. overnight, allantoic fluid was removed from each egg and tested for the presence of hemagglutinating substance by the addition of an equal volume (0.5 ml.) of 0.5 per cent chicken red cell suspension. Neutralization of infectious activity of the virus by serum was indicated by the absence of hemagglutination. The neutralizing activity of the serum is expressed in terms of the final dilution of serum in the particular

serum-virus mixture that would result in infection of 50 per cent of the embryos as calculated by the method of Reed and Muench (15).

Complement Fixation Test.—The method employed for testing complement-fixing activity is essentially the same as that in general use. The antigen used was the same one used in the other serologic tests. A 1:10 dilution of infected allantoic fluid was used as antigen after different quantities of the virus suspension were tested against twofold dilutions of antiserum of high titer to determine the optimal quantity of antigen for a test employing 4 hemolytic units of amboceptor, 1 full unit of complement, and a 1 per cent suspension of sensitized sheep red blood cells. The serum was heated in a water bath at 56°C., for 30 minutes, and then diluted serially in saline in twofold steps from 1:8 to 1:8192. Two identical series of serum dilutions were set up; to the first was added an equal volume of a 1:10 dilution of the virus antigen and to the second set was added a similar quantity of allantoic fluid from uninfected embryos. Suitably diluted complement was then added in a volume of 0.2 ml. After overnight incubation in the cold room at 1–4°C., the reaction mixtures were then placed in a 37°C. water bath for 10 minutes, 0.4 ml. of sensitized sheep red cell suspension added, and incubation continued for an additional 30 minutes. The test was read immediately and a positive reaction was considered to be one in which complete fixation of complement occurred; the end-point is expressed in terms of the initial dilution of serum before the addition of the reagents.

EXPERIMENTAL

In this section will be described the reactions observed and the immunologic findings in two experiments performed in groups of monkeys that were studied for a period of 1 year after inoculation or for as long as they survived within that interval. The essential distinction between the two experiments is the size of the inoculum; in the first a 1 ml. inoculum was used and in the second the volume administered was 0.1 ml. In each instance equal quantities of adjuvant (oil + emulsifying agent) and aqueous phase were blended together. As indicated in Tables I and II, the quantity of antigen contained in the respective doses of virus used in each experiment was varied over different ranges; the opposite extremes for the two experiments together are 100,000-fold apart. The immunologic data are presented in full in Tables I, II, and III; and, for particular emphasis, selected portions have been put in graphic form in Figs. 1 to 8.

In the experiment in which a 1 ml. inoculum was used, the vaccine was diluted in half-log steps; emulsified mixtures were prepared with the undiluted material and with the various dilutions through and including 10^{-2}. In the second experiment, in which 0.1 ml. was inoculated, tenfold dilutions of vaccine from undiluted to 10^{-4} were emulsified with mineral oil; groups of three or four animals were used in each experiment. Thus, monkeys of the second experiment that received the 0.1 ml. dose of the undiluted vaccine mixed with adjuvant received the same amount of antigen as did animals in the first experiment given a 1 ml. dose made up of the 10^{-1} dilution of vaccine plus adjuvant; similar overlapping of dosages of antigen used in the two experiments also occurred in groups given the next higher tenfold dilution in each series. The data obtained in these particular groups will be compared in the charts to follow.

Observations on the Site of Inoculation.—In no instance was it possible to distinguish by inspection or palpation between corresponding sites in uninoculated

TABLE I

Hemagglutination Inhibition Antibody Titers in Serum of Individual Monkeys at Intervals after Vaccination with Diminishing Quantites of Virus Combined with Adjuvant

Vaccine (1.0 ml. intramuscularly)	Interval post vaccination	Quantity of stock vaccine incorporated in inoculum* for each group														
		0.50 ml.			0.16 ml.			0.05 ml.			0.016 ml.			0.005 ml.		
Virus + adjuvant	Before	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	1 wk.	256	256	256	512	256	64	0	16	16	0	0	0	0	0	0
	2 wks.	2048	2048	2048	2048	4096	512	512	1024	1024	256	256	522	2048	512	1024
	4 wks.	2048	2096	4096	8192	4096	1024	512	1024	2048	256	512	512	2048	1024	1024
	6 wks.	8192	32,000	8192	8192	9182	1024	2048	2048	2048	256	512	512	2048	1024	512
	2 mos.	16,000	65,000	16,000	16,000	16,000	D	4096	1024	2048	1024	512	512	2048	1024	D
	3 mos.	D	65,000	32,000	65,000	16,000		4096	1024	2048	1024	512	1024	4096	512	
	4 mos.		32,000	32,000	65,000	8192		4096	512	2048	512	512	1024	2048	512	
	6 mos.		16,000	16,000	32,000	4096		4096	D	2048	512	512	1024	2048	256	
	9 mos.		D	16,000	16,000	2048		D		2048	512	256	1024	1024	256	
	12 mos.			8192	8192	1024				1024	256	256	512	D	128	
Virus + saline	Before	0	0	0				0	0	0						
	1 wk.	16	16	8				8	16	0						
	2 wks.	64	64	64				64	32	16						
	4 wks.	128	128	8				64	32	16						
	6 wks.	128	128	8				D	8	16						
	2 mos.	128	64	8					8	16						
	3 mos.	64	64	D					D	16						
	4 mos.	64	64							16						
	6 mos.	64	64							8						
	9 mos.	64	64							0						
	12 mos.	64	D							0						

* Containing mixture of equal parts of the virus dilution and adjuvant.
0 = <8; D = monkey died.

TABLE II

Hemagglutination Inhibition Antibody Titers in Serum of Individual Monkeys at Intervals after Vaccination with Diminishing Quantities of Virus Combined with Adjuvant

Vaccine (0.1 ml. intramuscularly)	Interval post vaccination	Quantity of stock vaccine incorporated in inoculum* for each group																			
		0.05 ml.				0.005 ml.				0.0 005 ml.				0.00005 ml.				0.000005 ml.			
Virus + adjuvant	Before	0	0	0		0	0	0		0	0	0	0	0	0	0	0	0	0	0	0
	1 wk.	32	32	32		0	64	32		64	0	0	0	0	0	0	0	0	0	0	0
	2 wks.	512	256	512		64	256	128		256	64	64	128	16	0	0	16	8	8	0	0
	4 wks.	1024	1024	1024		128	512	512		512	128	128	128	32	16	32	32	16	32	0	8
	6 wks.	1024	2048	2048		512	512	1024		1024	256	64	128	32	32	64	64	32	64	8	8
	2 mos.	1024	1024	2048		512	256	4096		512	128	64	128	32	16	64	64	32	64	8	8
	3 mos.	512	1024	D		1024	256	4096		512	128	64	128	32	16	64	64	16	128	0	16
	4 mos.	512	1024			512	128	8192		256	64	64	128	32	8	64	64	16	64	0	8
Virus + saline	Before	0	0	0	0	0	0	0	0												
	1 wk.	0	128	128	128	128	0	32	8												
	2 wks.	0	128	512	128	32	0	256	16												
	4 wks.	0	128	256	64	16	0	32	8												
	6 wks.	0	64	128	64	8	0	16	8												
	2 mos.	0	64	128	32	0	0	0	0												
	3 mos.	0	64	64	32	0	0	0	0												
	4 mos.	0	64	64	16	0	0	0	0												

* Containing mixture of equal parts of the virus dilution and adjuvant.
0 = <8; D = monkey died.

as compared with inoculated limbs in which either the 1 ml. or the 0.1 ml. dose was given. In the course of this and other experiments several animals died and at autopsy the inoculation site of the injected muscle was dissected for gross and microscopic examination. Depending in part upon the interval post vaccination, frequently the material injected could not be found in animals given the small dose; with the larger dose, however, it was easy to recognize the white emulsion distributed in fine droplets over wide areas, particularly along the muscle fibers. There was no gross evidence of acute inflammation; the droplets

TABLE III

Comparison of Antibody Titers Determined by Three Different Methods in One Monkey Vaccinated with Virus + Adjuvant and in Another Vaccinated with Virus + Saline

Interval post vaccination	PR8 virus + adjuvant*			PR8 virus + saline‡		
	Virus neutralization	Hemagglutination inhibition	Complement fixation	Virus neutralization	Hemagglutination inhibition	Complement fixation
wks.						
Before	0.0	0.0	0.0	0.0	0.0	0.0
1	0.9	2.1	0.0	0.6	0.9	0.0
2	2.2	3.0	1.2	0.6	1.5	0.0
4	2.5	3.3	1.5	1.6	1.8	0.0
6	4.1	3.6	2.1	2.5	1.8	0.0
8	4.3	3.9	2.5	2.5	1.8	0.0
12	5.2	4.2	2.9	2.5	1.5	0.0
16	5.3	4.2	2.9	2.5	1.5	0.0
26	5.2	3.9	2.4	2.5	1.5	0.0
39	5.1	3.9	2.4	2.4	1.5	0.0
52	4.9	3.9	2.1	2.2	1.5	0.0

Numerals represent negative $\log_{10}$ of serum dilution end-points expressed as the dilution of serum before the addition of other reagents incorporated in the serologic test.

* 1.0 ml. Intramuscularly of emulsion of equal parts of undiluted vaccine + mineral oil adjuvant.

‡ 1.0 ml. Intramuscularly of mixture of equal parts of undiluted vaccine + saline.

of emulsion were free and not bound by exudate. Microscopic examination revealed dense aggregates of mononuclear cells, the great majority of which were lymphocytic in appearance.

The observation that the inoculum was dispersed from the site of injection, presumably the result of muscular action, was especially pertinent to the original purpose of these experiments. It was observed that neither the oil, the emulsifying agent, nor the antigen appeared to be irritating; there was, therefore, ample opportunity for the emulsion injected to be dispersed into many small loci rather than be walled off by an immediate acute inflammatory response. The tendency for the emulsion to be dispersed when introduced intra-

muscularly, is in contrast to the tendency for it to remain as deposited when introduced subcutaneously. Under the latter circumstances, the inoculum becomes the content of a thin walled cyst, the lining of which is composed of the type of cellular reaction described above. Histologic studies on sections of muscle removed at intervals following inoculation will be reported in detail at a later date.

Immunologic Data.—All serum samples obtained at intervals following vaccination were tested for hemagglutination-inhibiting antibody; the data shown in Tables I and II, respectively, contain the results of the experiments with a 1

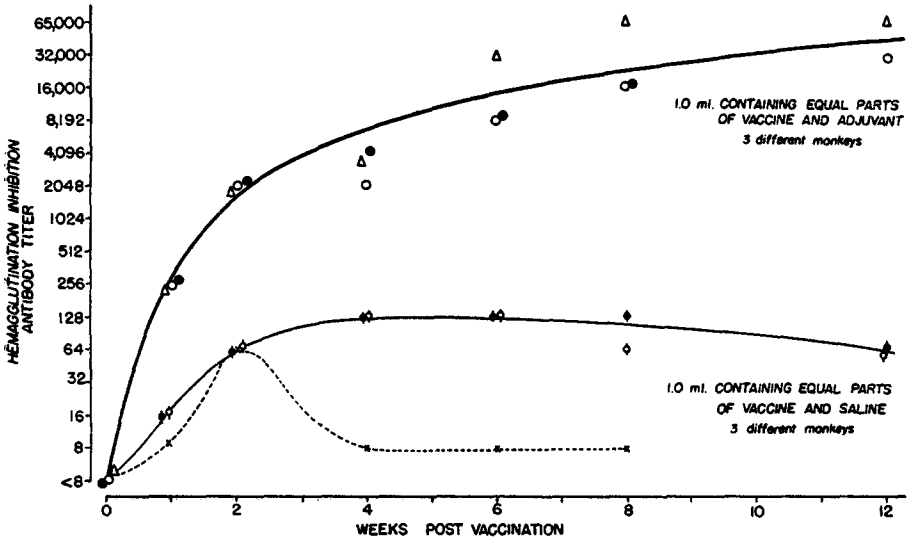

FIG. 1. Level of serum antibody at intervals post vaccination in monkeys inoculated intramuscularly with influenza A virus vaccine (PR8) mixed with mineral oil adjuvant or with saline.

ml. inoculum or a 0.1 ml. inoculum containing the different quantities of antigen. When one compares the course of development of antibody in monkeys that had received corresponding quantities with or without the adjuvant, it is clearly evident that much higher levels of antibody are induced in animals injected with the water-in-oil emulsion as compared with those that receive the vaccine without oil. Not only did the titers of antibody reach the high levels indicated but there was only a slight tendency toward decline in the 4 to 12 month interval post vaccination.

A curve is shown in Fig. 1 indicating the course of antibody development in three monkeys during the first 12 weeks following injection of the 1 ml. dose containing undiluted vaccine plus adjuvant; for comparison, curves for monkeys inoculated with a corresponding quantity of antigen mixed with saline are

also shown. In one of the three monkeys given the latter material, the level of antibody rose to a maximum of 1:64 at the 2 week period and then declined promptly. In the other two monkeys given virus in saline, the peak was reached in 4 weeks and the decline from a high of 1:128 became evident by the 12th week. This is in contrast to the continuing rise, sharply at first and then more gradually to levels of 16,000 to 65,000 at 8 to 12 weeks post vaccination in animals inoculated with virus plus adjuvant.

The data contained both in Tables I and II suggest that the maximal antibody level achieved diminishes as the quantity of virus contained in the inocu-

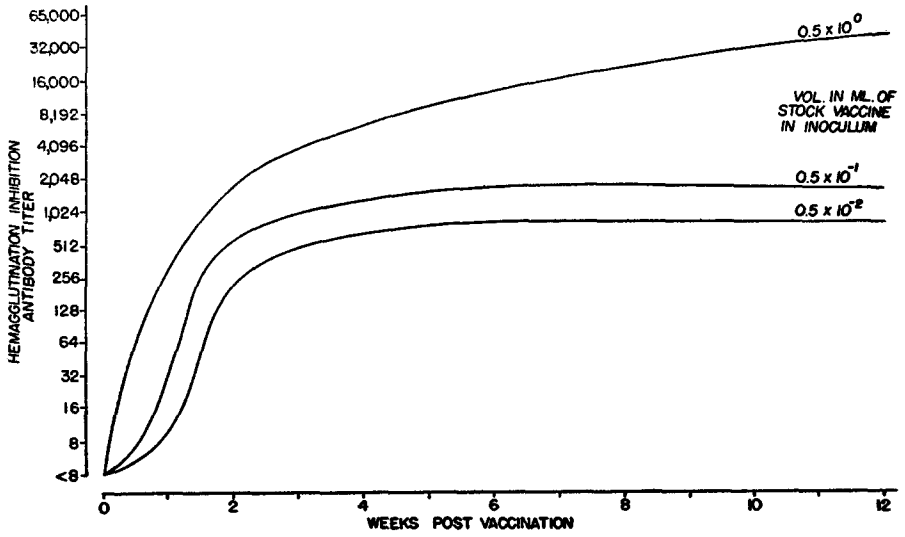

FIG. 2. Comparison of course of antibody development in monkeys inoculated intramuscularly with vaccines containing mineral oil adjuvant and different quantities of the nfluenza virus antigen (PR8).

lum is reduced. Before presenting a composite chart to illustrate the relationship between quantity of virus injected and level of antibody induced, it is desired to present the set of curves shown in Fig. 2 indicating the course of antibody development in the first 12 weeks in monkeys inoculated with a 1 ml. dose of emulsions containing different quantities of virus. These curves represent the best fit through the points for the three animals in each group given vaccines containing quantities of antigen that differed by tenfold steps. The data presented in Fig. 2 are intended merely to emphasize that relatively small doses of antigen, when incorporated with mineral oil, induce antibody formation described by curves similar to those drawn for vaccines containing much larger quantities of virus. It is of interest to note the different shapes of the curves in the early period post vaccination, especially with respect to the delay

in appearance of antibody following injection of the smaller doses. Data are insufficient at this time to warrant more than calling attention to the difference in the slope of the later part of the curve of antibody development in monkeys given the largest quantity of antigen as compared with the slopes of the curves describing the effect of the two smaller dosages.

Antibody Response to the Same Quantities of Antigen Contained in Different Volumes of Emulsion.—In the two experiments performed with graded dilutions of virus, groups of animals were inoculated with volumes that differed by tenfold, *i.e.*, 1.0 ml. of emulsion or 0.1 ml. At two levels, corresponding

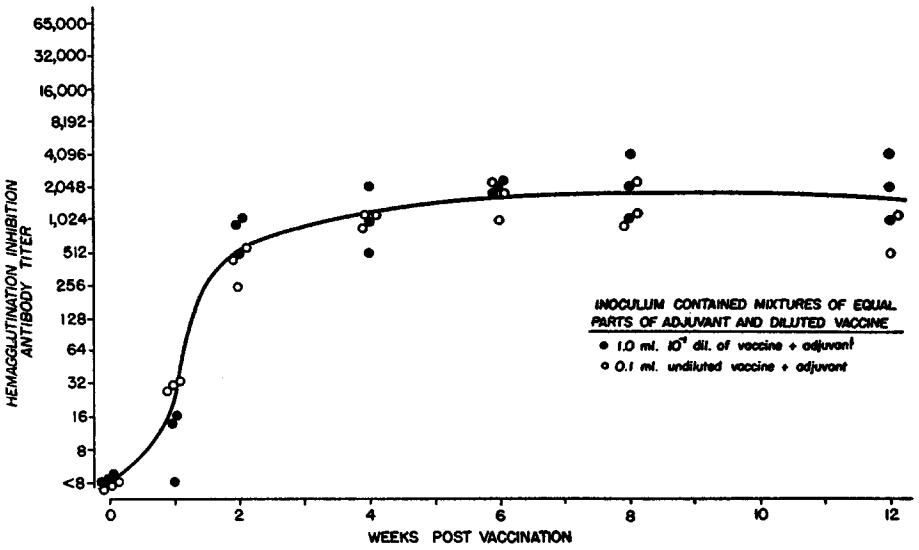

FIG. 3. Comparison of levels of serum antibody at intervals post vaccination in monkeys inoculated intramuscularly with corresponding amounts of influenza virus vaccine (PR8) contained in 1.0 ml. or 0.1 ml. of an emulsified mixture of equal parts of adjuvant and aqueous phase.

quantities of antigen were contained in the two different sized inocula. In all instances, however, the proportion of aqueous phase to oil and emulsifying agent was the same. From the data shown in Fig. 3, in which the two different doses are compared both containing the equivalent of 0.5×10^{-1} ml. of the stock vaccine, it is evident that the antibody response to 1.0 ml. or 0.1 ml. of emulsified vaccine cannot be distinguished when both inocula contain the same amount of antigen in the total volume inoculated. This conclusion gains further support from the data presented in Fig. 4 to be described in the section to follow.

Relationship between Concentration of Antigen in Virus-Adjuvant Mixtures and Quantity of Antibody Induced in Vaccinated Monkeys.—The relationship

between the quantity of virus injected into monkeys and the resulting titer of serum antibody is illustrated in Fig. 4; the influence of the amount of oil with which the antigen is incorporated is also illustrated in this figure. The antibody level for each monkey, 8 weeks post vaccination, is charted in relation to the quantity of antigen contained in the different inocula employed. The parallel sloping lines through the highest and lowest antibody levels induced by the different doses of vaccine are used to indicate the limits of variation in the relationship between quantity of antigen and degree of antibody response. The

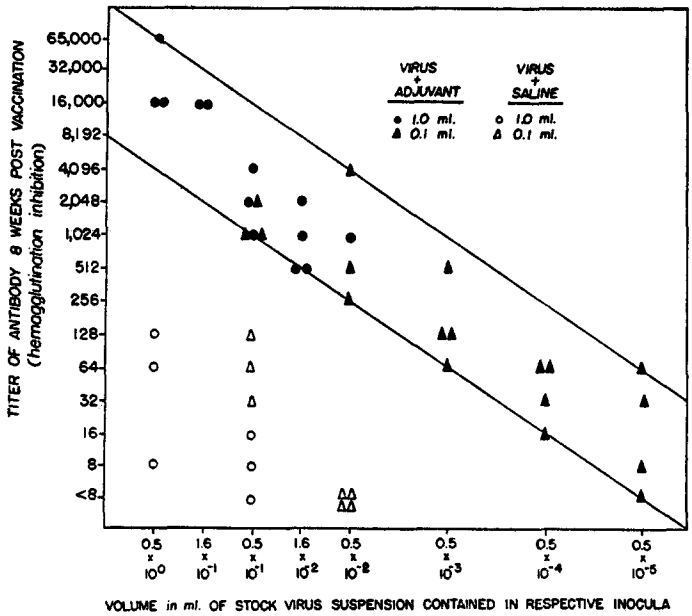

FIG. 4. Relationship between quantity of virus injected and titer of serum antibody in monkeys 8 weeks after inoculation of influenza A virus vaccine (PR8) mixed with mineral oil adjuvant. Comparison of results in animals inoculated with 1.0 ml. and 0.1 ml. intramuscularly.

slope of the area between the two lines is such that a 1000-fold difference in mean antibody level, induced by the extremes of dosage employed to construct Fig. 4, resulted from a difference of 100,000-fold in the quantity of virus administered; thus, a 1000-fold difference in quantity of antigen injected can be expected to influence the mean antibody level only 10-fold.

From Fig. 4 it is again evident that virus in saline was less antigenic than virus emulsified with the mineral oil. The stock vaccine, when diluted more than tenfold, and administered without adjuvant, resulted in little or no antigenic response so that hemagglutination-inhibiting antibody was not detectable 8 weeks post vaccination; in contrast, the same virus suspension when emulsified

with mineral oil before inoculation into monkeys induced a measurable amount of antibody after dilution as much as 100,000-fold.

In the discussion of the data in Fig. 3, it was pointed out that equivalent quantities of antigen when emulsified with mineral oil induced corresponding levels of antibody even though administered in volumes that differed by as much as tenfold. Thus, a small inoculum of emulsion containing concentrated virus is as effective in inducing and maintaining antibody formation as a large inoculum of an emulsion made with less concentrated antigen. This is further supported by the additional data in Fig. 4 which reveal the correspondence of 1.0 ml. or 0.1 ml. of emulsion containing 0.5×10^{-2} ml. of the stock vaccine as well as the 1.0 or 0.1 ml. volumes of the 0.5×10^{-1} ml. dose. Furthermore, the straight-line relationship throughout the entire range represented in Fig. 4 indicates the independence of volume of inoculum within the limits of 1.0 ml. to 0.1 ml. of emulsion. It should be recalled that the ratio of the aqueous phase to the other ingredients was constant. The principal variables were concentration of antigen contained in the aqueous phase, and volume of inoculum. It will be of interest to explore further the limits of this relationship but from a practical point of view it is noteworthy that a very small inoculum may be employed provided that it contains a sufficient amount of effective antigenic substance.

Comparison of Antibody Titers Measured by Hemagglutination Inhibition, Virus Neutralization in Ovo, and Complement Fixation.—It seemed important to establish whether or not the effect of the adjuvant upon the titer of antibody measured with the hemagglutination inhibition reaction had a corresponding influence upon the antibody measured by virus neutralization *in ovo*, and by complement fixation. Accordingly, all of the serum samples available from one monkey inoculated with 1 ml. of undiluted vaccine mixed with adjuvant were tested for titer of antibody by all three methods. Similar tests were performed on all serum samples of one monkey vaccinated with the same amount of virus mixed with saline. The data for both monkeys are shown in Table III and curves describing the development and persistence of antibody measured in the three different ways are shown in Fig. 5. It is clear that the adjuvant effect was evident with the virus neutralization and the complement fixation test as well as the hemagglutination inhibition test. It appears from the pair of animals involved in the present study that the virus-adjuvant mixture induced titers of virus-neutralizing antibody and hemagglutination-inhibiting antibody that persisted at levels approximately 1000-fold higher than those induced by the corresponding amount of virus in saline. In some animals inoculated with the more concentrated virus suspension and adjuvant, minimal quantities of complement-fixing activity were detected in tests employing normal allantoic fluid as antigen. The titer for influenza antibody was so very much higher as to obviate any difficulty in interpretation.

Although complement-fixing antibody was readily demonstrable in the serum

of the monkey given virus fortified with adjuvant, this antibody was not found in the serum of the monkey given virus in saline. The highest complement-fixing titer that appeared in the virus-adjuvant vaccinated monkey was approximately

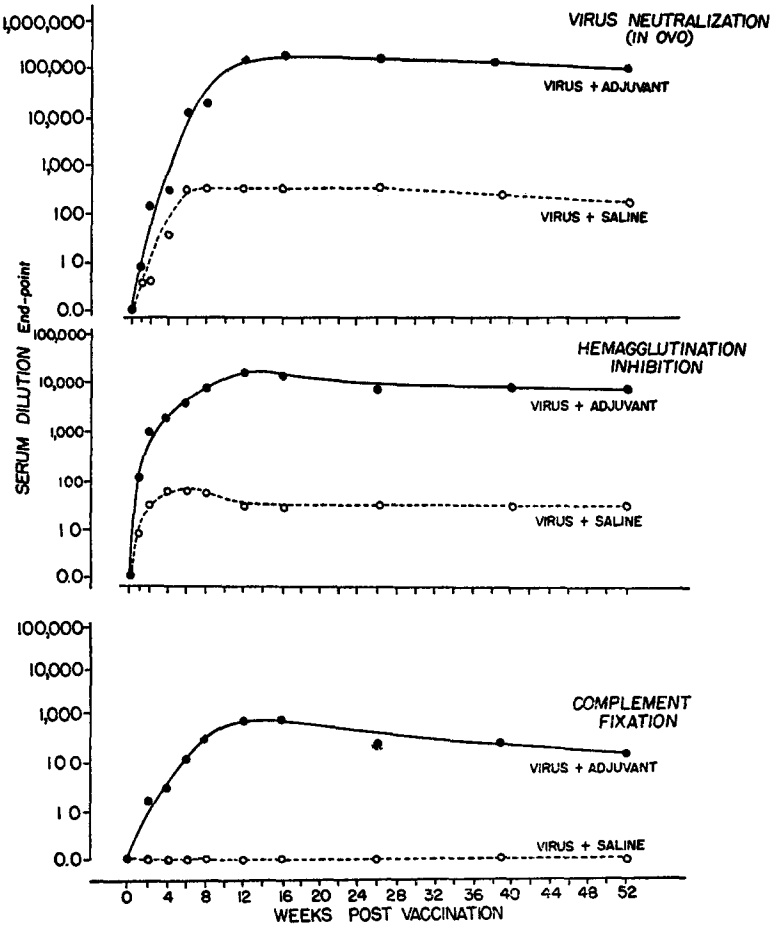

FIG. 5. Antibody levels as measured by virus neutralization (*in ovo*), hemagglutination inhibition, and complement fixation at intervals up to 1 year in one monkey inoculated with virus + adjuvant and one with virus + saline.

1:1000; it was essentially zero in the virus-saline vaccinated animal. Since the difference between titers for hemagglutination-inhibiting and virus-neutralizing activity in monkeys vaccinated with virus and adjuvant or virus and saline was approximately 1000-fold, it would seem that failure to detect complement-fixing antibody in the monkey vaccinated with virus in saline may have been due to quantitative factors related to the relative sensitivity of the complement

fixation system for measuring antibody as compared with the other two methods. However, it is also possible that all three activities are different and that qualitative factors are responsible for the quantitative differences that are observed. Although considerably more evidence is needed to support one hypothesis or another to explain the relationship of the antibody activities measured by the three different methods, it is of interest to examine in greater detail the data derived in these experiments as they may bear upon the question of relationship of the three antibody activities.

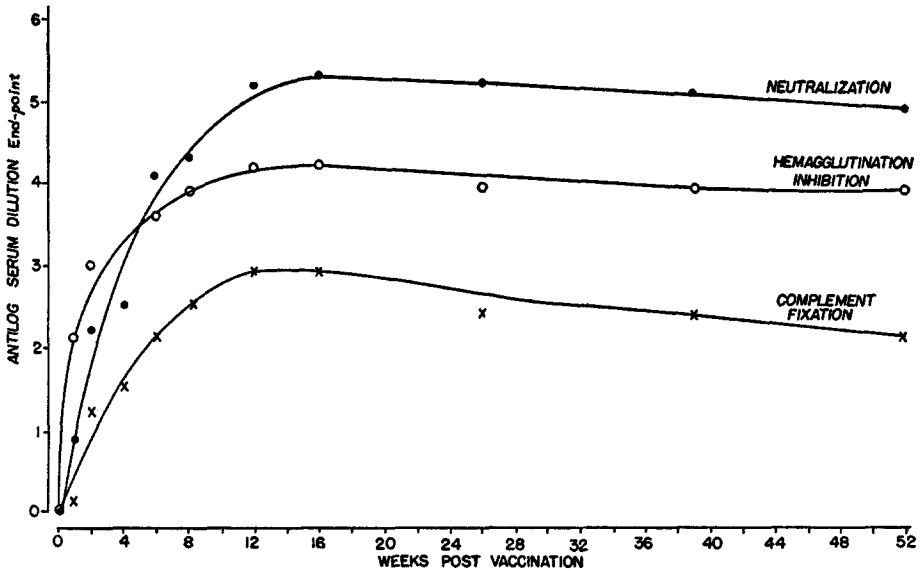

FIG. 6. Comparison of curves showing development and persistence over 1 year of antibody measured by virus neutralization (*in ovo*), by hemagglutination inhibition, and by complement fixation in a monkey vaccinated with virus + adjuvant.

Curves describing the development and persistence of the three different antibody activities in the serum of the virus-adjuvant vaccinated monkey are shown together in Fig. 6. It is obvious that at about the 6th week post vaccination there is a crossing of the curves describing the development of hemagglutination-inhibiting antibody activity and virus-neutralizing activity. In the early period the hemagglutination-inhibiting titer of the serum has a higher value than the virus-neutralizing titer and in the later period the reverse is true. Even though numerical values for complement-fixing titers are lower than for the other antibodies, it appears that the curve expressing the development of complement-fixing antibody does not parallel either of the other two curves.

In order to determine the trend and the significance of the apparent differences in the rates of development of the three antibody activities, a factor was

derived for expressing the difference between the titers of serum antibody activity measured by two methods; the factor merely represents the difference between the log values for the two serum antibody titers and is charted in Fig. 7 in relation to the time post vaccination that the serum was obtained. Fig. 7 shows the relationship to time post vaccination of the factor for the log hemagglutination inhibition titer minus the log virus neutralization titer. If both antibody activities had developed at the same rate, the difference factor would be the same throughout the period of antibody development; the points would

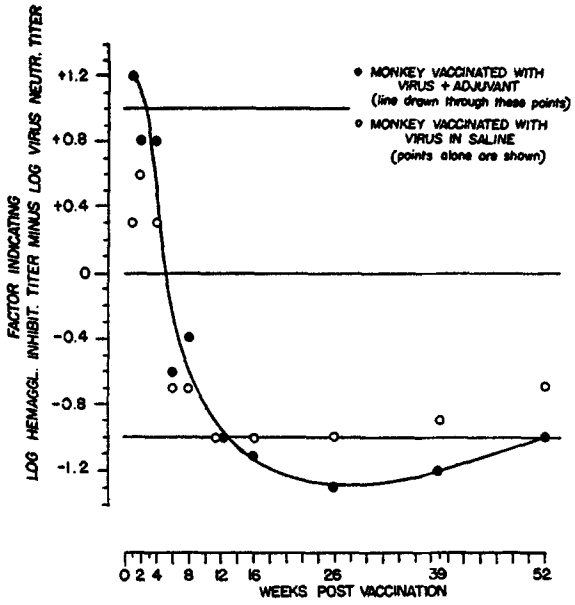

FIG. 7. Curve indicating the effect of interval post vaccination upon the difference between log values for two antibody activities as an expression of the difference in rate of development and decline of hemagglutination inhibition and virus-neutralizing antibody.

then have described a line parallel to the horizontal lines drawn in Fig. 7. It is evident that the difference factor, plotted as a function of time does not describe a horizontal line in the first 12 week period post vaccination. In this period the hemagglutination-inhibiting antibody activity developed more rapidly than virus-neutralizing activity; in the later period the indications are that the hemagglutination-inhibiting titer may have declined more slowly. The latter conclusion might be questioned in view of the very slight degree of variation from the parallel line drawn at 1.0 log on the negative side of the scale were it not for the consistency of the trend both in the monkey vaccinated with virus and adjuvant and in the animal given virus in saline. Fig. 8 indicates in a similar manner the independence of the factors for hemagglutination-inhibiting ac-

tivity and complement-fixing activity; corresponding data are available indicating the independence of the virus-neutralizing and complement-fixing activities.

The results of other experiments on the question of the relationship of the different antibody activities of the serum of animals immunized with influenza viruses are to be reported. The observations described here confirm still others made earlier (16, 17), in the course of studies on immunization of mice in which it was found that hemagglutination-inhibiting activity appeared more rapidly

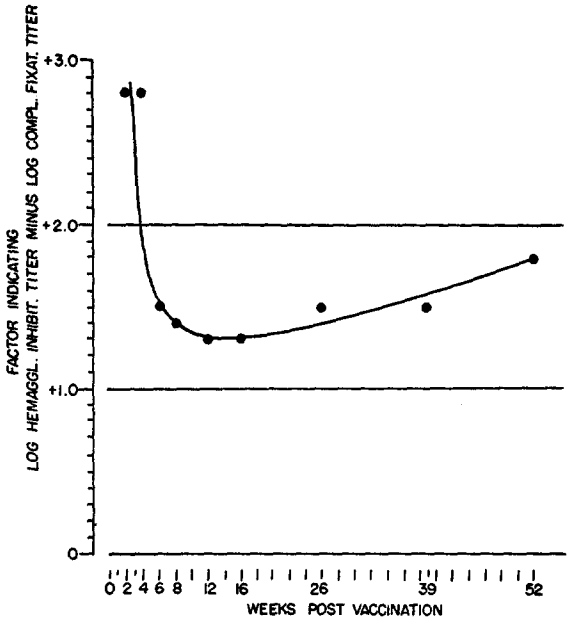

FIG. 8. Curve indicating the effect of interval post vaccination upon the difference between log values for two antibody activities as an expression of the difference in rate of development and decline of hemagglutination inhibition and complement-fixing antibody.

than virus-neutralizing activity. The latter observations (16) were made not only with another animal species but with a type B strain (Lee) as well as with type A strains. Walker and Horsfall (18), employing a different approach, have presented evidence indicating that the virus-neutralizing and hemagglutination-inhibiting activities of serum could be differentially absorbed by interacting graded quantities of virus with immune serum; they concluded that the two antibodies are not identical.

Thus, it has been established that the enhancement in antibody formation induced by vaccination with virus emulsified with mineral oil influences the virus-neutralizing and the complement-fixing activities as well as hemaggluti-

nation inhibition activity of the serum of vaccinated animals. Careful analysis of the rates of development and decline of the three different properties of serum indicates that they are independent functions and if not associated with different components of the serum separable one from the other, they are at least induced by different components of the antigenic complex of which the influenza virus is composed. It is apparent that much more need be done to clarify our understanding of the underlying factors and that this can probably be done only through the successful dissection of the influenza viruses in a manner similar to that which has been applied to the separation of the various antigenic components of many bacteria and of certain viruses, notably vaccinia. Studies are in progress to determine which, if any, of the antibodies are directly related to immunity to infection.

DISCUSSION

In the studies here reported untoward local reactions were not observed following inoculation of monkeys with influenza virus vaccine with a mineral oil adjuvant. Nevertheless, there occurred a marked enhancement in antigenic effect. It is desired, therefore, to emphasize the particular features of the present investigations that are different from those reported by others in which inoculation of mineral oil adjuvant with or without acid-fast bacilli resulted in undesirable reactions at the site of inoculation.

It is believed that several considerations account for the differences in the observations of the present and previous studies. Contributing factors include the use of an emulsifying agent that is non-irritating, a mineral oil of low viscosity, and an antigen, at least in the concentrations employed, that did not induce an inflammatory reaction; a matter of considerable importance was the introduction of the inoculum intramuscularly. The use of the muscular rather than the subcutaneous depot promotes, by muscular action, the dispersion of the emulsion into numerous small droplets; when introduced subcutaneously the inoculum tends to remain localized with resulting nodule or cyst formations. Acid-fast bacilli were not incorporated into the emulsions used in these studies.

The immunologic phases of these investigations indicate that rather minute quantities of antigen, when incorporated with mineral oil, are still effective in antibody formation. Thus, approximately 1,000- to 10,000-fold less virus, beyond the point of antigenic extinction of virus in saline, is still active antigenically when combined with mineral oil. The reason for the much greater immunologic efficiency of virus plus adjuvant is suggested by several diverse observations. Histologic findings and the results of certain other investigations employing radioactive isotopes indicate not only a prolonged retention of the antigen at the inoculation site but also the aggregation around the oily droplets of large numbers of lymphocytes and other mononuclear elements. From what is known about the mechanism of antibody formation, it would appear that

certain chemical and physical attributes of the mixture injected exert a tropic influence upon the wandering cells active in antibody formation. It may be postulated that, as a result of the accumulation around the emulsion of cells which probably play a part in antibody formation, there is created what might be referred to as an antibody producing organ. In other words, it is conceived that the antigen in oil is retained at the site of inoculation and antibody producing cells are brought to it by the chemotropic influence of the constituents of the emulsion. An aqueous suspension of virus is absorbed rapidly, dissipated throughout the body, and destroyed or taken up by various cells only a few of which may be active in antibody formation. According to this hypothesis, it is readily apparent that whatever quantity of antigen is administered would be utilized more efficiently if injected as an emulsion with a suitable adjuvant rather than in an aqueous menstruum.

The demonstration that antibody activities measured by three different methods develop at different rates and that each probably declines at a rate independent of one another, makes it all the more imperative to establish which if any of these activities parallel the level of immunity that develops after vaccination. It is clear that one can no longer speak of influenza antibody; it is necessary to refer to the method used in its determination.

The methods employed for characterizing the *dimensions of antigenicity* of the virus studied in these experiments may be used for establishing similar facts for other viruses that might be considered for incorporation in a vaccine. Employing techniques similar in principle to those used in these studies, it is already apparent that vast differences exist in the antigenic capacity of different strains of influenza virus and also among different antigens that may be present in a single mixture. The practical considerations involved in immunization of man or animals call for quantitative expressions of level and duration of persistence of antibody or immunity. The results of the experiments here reported indicate that it may be possible with the aid of mineral oil adjuvants to produce effective levels of antibody and, perhaps, immunity by the use of much smaller quantities of antigen than heretofore considered. Under the latter circumstances, it will become desirable to know something about the antigenic extinction end-point of a particular antigen when combined with mineral oil so as to permit the development not only of the most effective preparation for immunization but the safest and most efficient. It is equally evident that by reducing the quantity of any one antigen in a mixture that might be used for immunization, it might then be possible to incorporate other antigens as well and then ultimately reduce the number of immunizing injections necessary to create and maintain immunity for the various infectious diseases to which man and animals are susceptible. The extension of these studies in man and other animals is under way and the results of these investigations are soon to be reported.

SUMMARY

Untoward reactions at the site of inoculation were not observed in monkeys vaccinated with influenza virus incorporated in a water-in-oil emulsion without acid-fast bacilli.

Studies were then made to measure some of the dimensions of antigenicity of these emulsions to evaluate the extent of the immunologic adjuvant effect. This included measurements of height and persistence of the antibody response to inoculation and measurements of the extent to which the vaccine could be diluted and still induce antibody formation; *i.e.*, antigenic extinction. In addition, comparisons were made of the rates of development of hemagglutination-inhibiting, virus-neutralizing, and complement-fixing antibody activities to determine the relationship among these three properties of the serum of immunized animals.

It was found that levels of antibody many fold higher were induced by the virus-adjuvant mixtures as compared with virus in an aqueous menstruum, and that the level of antibody induced was related to the quantity of antigen incorporated in the emulsion. The stock vaccine when emulsified could be diluted 100,000-fold and was still active in antibody formation whereas a 100-fold dilution of the antigen without emulsification was essentially ineffective. Equivalent quantities of virus in 0.1 ml. or 1.0 ml. of emulsion induced antibody responses that were indistinguishable with respect to level or persistence.

In comparing the course of antibody development it was found that hemagglutination-inhibiting, virus-neutralizing, and complement-fixing antibodies develop at different rates; careful analysis of the data derived from the present study together with other observations warrant the conclusion that these antibody activities are not present in constant proportion and are independent of one another. The implications of this observation and of the others mentioned above are discussed.

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